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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:) For: In-Situ Extraction and Assay
) Method
Jeffrey Kenneth Horton)
) Group Art Unit 1643
Serial No: 09/027,654)
) Examiner: To Be Determined
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TRANSMITTAL OF PRIORITY DOCUMENT

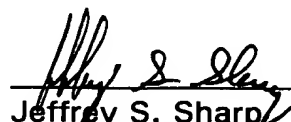Assistant Commissioner for Patents
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Sir:

Enclosed herewith are certified copies of Great Britain Patent

Application No. 9715704.4, filed July 24, 1997 and European Patent

Application No. 97301398.0, filed March 3, 1997, upon which priority of

the instant application is claimed under 35 U.S.C. §119.

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Patentanmeldung Nr.   Patent application No.   Demande de brevet n°

97301398. 0

Der Präsident des Europäischen Patentamts:
Im Auftrag

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   In-situ cell extraction and assay method

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IN-SITU CELL EXTRACTION AND ASSAY METHOD

FIELD OF THE INVENTION

The present invention relates to the field of immunoassays. The invention provides a simple and convenient, single stage, single vessel cell extraction and assay method which is suitable for the extraction and measurement of a range of different types of analyte which occur as cellular components. The invention also relates to kits of reagents suitable for performing cellular extraction and measurement as a single stage, single vessel process.

BACKGROUND TO THE INVENTION

1.   Immunoassay Technology

A number of techniques are known and have been described for the measurement of small quantities of biological materials. Of these techniques, the area of immunoassays has been extensively reviewed and the technique forms the basis of many commercially available assay kits.

For more than thirty years, immunoassay has been the method of choice for measuring low analyte concentrations in complex biological fluids. The procedure is equally applicable to the measurement of low molecular weight compounds such as drugs, steroids and the like, as well as large molecular weight compounds such as protein molecules. The technique combines sensitivity and specificity. Immunoassays are used in basic biological research to investigate the physiological and possible pathological role of a wide range of potent biologically active substances, including cyclic nucleotides, prostglandins, leukotrienes, growth factors, steroid hormones and cytokines. Such research often leads to the identification of new therapeutic agents. Immunoassays are

often used in the pharmaceutical industry in many aspects of drug development processes. These range from drug screening, toxicological, pharmacological and pharmacokinetic studies, through to clinical trials. Immunoassays have had their greatest impact in the area of clinical

5 diagnostic tests. The technique has been employed for many years in hospital clinical biochemistry laboratories to diagnose disease and metabolic disorders. The technique was introduced in 1959 by Berson & Yalow. (Yalow, R.S. and Berson S.A., Assay of plasma insulin in human subjects by immunologic methods, Nature, (1959), 184, 1684). The

10 combination of a signal which could be detected and a protein molecule (an antibody) which binds specifically and avidly to the analyte of interest lies at the heart of all immunoassay procedures. Assay designs have proliferated over the last thirty years, as have the different types of signal reagents and detection systems. Sophisticated instruments with associated

15 computer hardware have been developed with the aim of increasing sample throughput. Further background information relating to immunoassay techniques can be found in 'The Immunoassay Handbook, (Wild, D.G. Ed, Stockton Press, New York, (1994), which deals with many of the concepts associated with immunoassay technology which are

20 pertinent to the present invention. It considers, for example, competitive (also termed 'labelled analyte' or 'limited reagent') and immunometric ('labelled antibody' or 'reagent excess') systems.

The earliest methods were those which involved a step of separating the bound analyte from the free, in order to be able to measure

25 the amount of bound analyte. Various separation methods have been described, including charcoal absorption, ammonium sulphate precipitation, magnetic particles ('Amerlex™'), etc. More recently, solid supports have been utilised for immunoassay procedures, including the walls of microtitre well plates.

30 A more recent development has been the introduction of

homogeneous radioimmunoassay technology, notably the technique of scintillation proximity assays (SPA) covered by US Patent No. 4,568,649. Scintillation proximity assay is a radioisotopic assay technique which has gained wide acceptance in recent years, and is applicable to

5   radioimmunoassays, as well as to radio-receptor and enzyme assays. The technique relies on the observation that β-particles emitted from radioisotopes will travel only a limited distance in an aqueous environment (in the case of tritium β-particles, this is 1.5μm), before the energy is dissipated. In SPA, the target of interest is immobilised to a small

10   microsphere containing scintillant. When a radioisotopically labelled molecule is brought into close proximity with the microsphere, β-particle energy is transferred effectively to the scintillant, thereby causing the emission of light. Labelled molecules which remain free in solution are undetected because they are too distant from the scintillant-containing

15   microsphere. In a typical radioimmunoassay, the microsphere is coated with a capture moiety, such as protein, A, or secondary antibodies, such as donkey-anti rabbit, sheep-anti-mouse antibodies. A sample, containing or suspected of containing the analyte (i.e. antigen) to be tested, is incubated in the presence of an antibody specific for that analyte, together with a

20   quantity of a radiolabelled analyte. The antibody/analyte complex is captured by the secondary antibody and is detected by the emission of light. Any labelled antigen which remains unbound by the antibody, will be free in solution and be undetected. The assay therefore requires no separation step and the protocol has fewer pipetting steps compared with

25   conventional, i.e. separation-based radioimmunoassays. It has been shown that in SPA-based assays, there is often an increase in assay precision and reproducibility, compared with traditional separation-based assays. Another advantage lies in the potential for increased sample throughput and capability for automation. (Cook, N.D., Drug Discovery

30   Today (1996), 1, 287-294). The application of SPA to RIA methodology is

not restricted to particular analytes or to types of molecule and in principle the technique can be applied in place of traditional separation-based assays. Some examples of RIAs developed using SPA are shown in Table 1.

Table 1. Examples of Radioimmunoassays Developed using SPA

Assay	Reference
Cyclic AMP	Horton JK & Baxendale PM (1995), In: Methods in Molecular Biology,41,pp. 91-105, Eds. Kendall, DA and Hill, SJ, Humana Press Inc, Towota, NJ
Cyclic GMP	Heath R Bryant B & Horton JK (1992), In: The Biology of Nitric Oxide. Part 2. Enzymology, Biochemistry and Immunology pp. 98-102, Eds. Moncada , S et al. Portland Press
6-Keto-Prostoglandin F1 alpha	Baxendale PM et al (1990) In: Advances in Prostaglandins, Thromboxane and Leukotriene Research, 21, pp. 303-306, Eds. Samuelsson, B. et al, Raven Press
Acyclovir	Tadepalli, SM, Topham, PA & Quinn, RP. (1990) Clin. Chem. 36, 1104
Platelet Activating Factor	Sugatani , J et al (1990), Life Sciences, 46,1443-1450
Abscisic Acid	Whitford, PN. & Croker, SJ. (1991) Phytochemical Analysis, 2, 134-136
Androstenedione	Fiet, J. et al (1991) Clin. Chem., 37,293
Ranitidine	Linacre, P. & Morris, SE, (1992), In: Bioanalytical Approaches for Drugs, including anti-asthmatics and metabolites, 22, pp.325-326 Eds. Reid, E. & Wilson, ID. Royal Society of Chemistry, London

More recently, alternatives to scintillant-containing beads (fluomicrospheres) have been described for use in proximity assays. PCT Application No. WO 90/03844 (Wallac) discloses a microtitre well plate intended for binding assays. The sample plate is produced from a transparent scintillant-containing plastic by means of a vacuum thermoforming or injection moulding process. In principle, the walls of the microtitre well plate can be coated with a binding compound for the purpose of performing *in vitro* binding assays using radiolabelled reactants.

PCT Application No. WO 94/26413 discloses an apparatus and a method for studying cellular biochemical processes in real time. In one aspect, the application describes a multiwell plate, such as a microtitre well plate, in which the base of the plate is formed from a scintillant plastic material and the walls are formed from an opaque plastic material, the wells of the plate being adapted for the attachment or growth of cells. The scintillating microplates are designed for use in the real-time analysis of a wide spectrum of cell associated phenomena, and applications have been demonstrated in transport, cell motility, uptake, metabolism and other cell based processes. Cytostar™ scintillating microplates form the basis of a new technology introduced by Amersham International plc, for the study of cellular processes. In other applications, the scintillating microplates can be used for *in vitro* assays, for the measurement of ligands, analytes, etc. In this format a binding compound is bound to the walls of the microtitre well plate for reaction with label and analyte.

As an alternative to radioisotopic methods for performing immunoassays, non-radioactive systems have been introduced. Today, enzymes are the most widely used tracers. When in combination with colourimetric end-points, they provide highly sensitive, robust, precise, accurate and convenient immunoassays. A major breakthrough came with the introduction of ninety-six well microtitre plates. Inexpensive automatic colourimetric multiwell plate readers are available. A number of other non-

isotopic labels have been described, of which luminescent and fluorescent labels are the most popular.

2.   Cell Extraction Methods

5   Traditional methods for immunoassay depend on obtaining the samples in a sufficiently suitable state, i.e. sufficiently free from interfering factors . Usually this will involve a cellular extraction method. Numerous procedures are described which detail the extraction of intracellular molecules from cells. Typically these methods involve acid,

10   solvent or solid phase methods to accomplish cell lysis and extraction of the molecule of interest. Methods for performing such extractions can be found in several publications. Further background information relating to cell extraction methods can be found in a review article by Goldberg & O' Toole. (Goldberg, ND & O'Toole, AG (1971); In: Methods of Biochemical

15   Analysis, 20, Ed Glick D. pp 1-39 Interscience Publishers, Wiley, London)
     Examples of cellular extraction methods are as follows.

2.1   Solvent Extraction (Horton & Baxendale, 1995; See Table 1 for Reference)

20   Ice-cold ethanol is added to cell cultures to give a final suspension volume of 65% (v/v) ethanol and the suspension allowed to settle. The supernatant is aspirated into test tubes, the remaining precipitate washed with ice-cold 65% (v/v) ethanol and the washings added to the appropriate tubes. The extracts are centrifuged at 2000g for

25   15 minutes at 4°C and the supernatant transferred to fresh tubes. The combined extract is then dried overnight, either under a stream of nitrogen at 60°C, in a vacuum oven for 8 hours, or in a centrifugal evaporator on a high temperature setting for 4 hours.

     In this procedure, there is a possibility of overdrying, and this

30   can result in difficulty in reconstituting the samples. The dried extracts are

dissolved in a suitable volume of assay buffer before analysis.

2.2   Acid Extraction

Hancock *et al*, (J. of Receptor & Signal Transduction
Research, 1995, 15, 557-579) describe an acid extraction method for
intracellular molecules in which 0.2M hydrochloric acid is added to cells,
and each separate sample is vortex mixed for 1-2 minutes. The sample is
carefully neutralised to a pH that is compatible to the immunoassay, using
stepwise addition of 10μl aliquots of 2.5M sodium hydroxide, care being
taken to measure the pH of the sample after each addition of alkali. This
step is particularly critical, as the use of a non-optimal pH with an
immunoassay can result in inaccurate measurement or non-measurement
of analyte in the samples.

An alternative approach is described by Steiner, (In: Methods
of Hormone Radioimmunoassay ,1979, Eds Jaffre, BM, & Behrman, HR
pp. 3-17 Academic Press, New York), in which cell samples are
homogenized in cold 6% (w/v) trichloroacetic acid at 4°C to give a 10%
(w/v) slurry. The sample is centrifuged at 2000g for 15 minutes at 4°C.
The supernatant is reserved and the pellet discarded. The supernatant is
washed four times with five volumes of water-saturated diethyl ether,
discarding the upper layer after each wash. The aqueous extract is
lyophilized overnight or dried under a stream of nitrogen at 60°C overnight
and the dried extract dissolved in a suitable volume of assay buffer before
analysis.

2.3   Solid Supports (e.g. Ion Exchange or 'Amprep' columns)

A protocol for the extraction of intracellular molecules by ion
exchange chromatography, using disposable minicolumns, has been
described previously (Horton & Baxendale, 1995; see Table 1 for
Reference). The columns (for example, ion exchange SAX columns) are

used with a vacuum manifold and a vacuum pump. The columns are prepared by applying a vacuum and rinsing with 2ml 100% methanol, followed by washing with 2ml of water, taking careful precautions so as not to allow the solid support to dry, or to allow the flow rate to exceed

5   5ml/minute. The cultured cells are applied directly to the column and washed with 3ml 100% methanol. Three millilitres of acidified methanol (prepared by diluting concentrated hydrochloric acid to 0.1M with absolute methanol) is added to the column and the eluate collected. The fractions are dried using a stream of nitrogen or in a vacuum oven overnight (see

10   above). The samples are reconstituted in assay buffer, as described above, before assay.

2.4   Detergent methods

Other methods are known for the extraction of nucleic acid

15   samples and nucleotides such as ATP. For example, Lundin and Anson (PCT WO 92/12253) describe a method for extracting an intracellular component in which bacterial cells are lysed with a detergent which is subsequently neutralised by addition of a cyclodextrin. A cellular component (e.g. ATP, DNA or RNA) liberated is subsequently measured

20   using biochemical or molecular biology (non-immunoassay) techniques such as the firefly luciferase and polymerase chain reaction assays. No reference is made in this patent application to one-step assays, homogeneous immunoassays, including scintillation proximity assay methods, or separation based immunoassay techniques.

25   The use of cyclodextrins to remove surfactants from solutions and surfaces and surfaces has been described previously in European Patent Application EP 301 847 (P. Khanna and R. Dworschack). According to this patent application, surfactants can be removed and cleaned from solutions and containers used in biochemical reactions by

30   immobilised cyclodextrins. EP 286367 (Khanna *et al*) describes

cyclodextrins as neutralisers of surfactants used as storage stabilisers for enzymes which are used as tracers in enzyme immunoassays. In a review, various applications of cyclodextrins in biological and chemical reactions have been described. (J. Szejtli, Cyclodextrins in Diagnostics,
5   Kontakte [Darmstadt] 1988 [1]. 31-36).

The use of cyclodextrins, to neutralise surfactants added as extractants to release intracellular molecules, in a simple, single-step extraction and measurement immunoassay system has not been described previously.

10   All of the above prior art methods for immunoassays suffer from a number of disadvantages, including:

i)   Unable to process large numbers of cells samples

ii)   Time consuming

iii)   Labour intensive

15   iv)   Prone to errors because of the large number of steps

v)   The need to remove the cell extraction reagent before further processing and measuring can take place. If this is not carried out, then accurate measurements may not take place, or, indeed, could result in total assay inhibition, and therefore measurement of the substance in the
20   cellular extract is prevented.

In all of the traditional methods of sample preparation for radioimmunoassay, it has hitherto been necessary to perform separate lysis and extraction processes in order to obtain samples in a suitable form for subsequent measurement. The prior art methods therefore involve
25   three separate processes which must be carried out sequentially, thereby adding to the time and cost of each immunochemical assay. In addition none of the prior art methods for the assay of intracellular components would be amenable to high throughput screening methods which are necessary if large numbers of samples are required to be processed. In
30   this specification, data is presented whereby addition of a cellular lysis

reagent to an immunoassay system, results in inhibition of antigen:antibody binding; and the inclusion of complex carbohydrates, such as cyclodextrins, restores the antigen:antibody binding event. In the preferred embodiment of the invention, 1% DTAB (dodecyl trimethyl

5   ammonium bromide) is employed as a cellular lysis reagent (which inhibits antigen:antibody binding) and 2.5% alpha cyclodextrin is used as a sequestration reagent restoring antigen:antibody binding. These reagents, together with homogeneous immunoassay techniques, have enabled, for the first time, the establishment of a concerted one stage, single pot,

10   cellular lysis and immunoassay system for the accurate measurement of intracellular molecules. Clearly, the method described in this patent application can be readily adapted for use with traditional separation, non-homogeneous immunoassays, and also two-stage methods whereby cells are cultured in separate vessels from those used to carry out the

15   immunoassay measurements. In this case, cellular lysis and sequestration reagents are used in combination with cell samples, labelled and unlabelled specific binding partners as described below (see Description of the Invention).

Thus, a novel, convenient and rapid method for the extraction

20   and quantitation of target molecules is described here, which permits the growth of cells, the extraction of intracellular components and the subsequent assay of such components to be carried out in the same vessel. The technique is simple to perform and can be carried out with little technical intervention. Since few manipulations are necessary, the

25   procedure is fully amenable to robotic automation

DESCRIPTION OF THE INVENTION

The invention provides a method of assaying for an analyte which method comprises the steps of:

30   i)   mixing a sample of cells possibly containing the analyte with

a cell lysis reagent to provide a cell lysis fluid,

ii)   mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

5   iii)   and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

The analyte is a cellular component whose nature is not material to the invention. Any cellular component for which a specific

10   binding partner is available can in principle be utilised in the invention. Typical specific-binding partner combinations suitable for use with the invention may be selected from: hapten-antibody, ligand-receptor, DNA-DNA, RNA-RNA, DNA-RNA, biotin-streptavidin, protein-antibody, peptide-antibody, and polypeptide-antibody interactions. Preferably the

15   specific binding assay is a protein-binding assay or particularly an immunoassay. Typical cellular components include proteins, peptides, second messengers such as cyclic AMP and cyclic GMP, hormones, steroids, peptides, prostaglandins, inositol phosphates, intracellular cytokines chemokines and leukotrienes.

20   The cell lysis reagent is preferably a detergent, that is to say a surface active agent which may be cationic, anionic, zwitterionic or non-ionic. Examples of suitable detergents include dodecyl trimethyl ammonium bromide (DTAB); cetyl pyridinium chloride (CPC); benzethonium chloride (BZC); sodium dodecyl sulphate (SDS), and N-

25   dodecyl-N,N-dimethyl-3-ammonio-1-propane sulphonate (DDAPS). DTAB, CPC and BZC are cationic surfactants; DDAPS is a zwitterionic surfactant and SDS is an anionic surfactant. The use of these detergents as cell lysis agents is well known in the field. Typical concentrations are in the range of 0.4 - 4% by weight on the weight of the cell lysis fluid. If too little detergent

30   is used, then cell lysis may be slow or incomplete. In addition to lysing

cells in order to release an intracellular component into a cell lysis fluid, the detergent may also adversely affect the binding of that intracellular component to its specific binding partner added in the course of step ii) for assay. The sequestrant is used to inhibit or annul that undesired adverse effect.

A key feature of the invention is the use of a sequestrant for the cell lysis reagent. The sequestrant acts to prevent the cell lysis reagent from adversely affecting a binding reaction between the analyte and its specific binding partner. The sequestrant may do this e.g. by chemically reacting with the cell lysis reagent or by physically absorbing it. Preferred sequestrants are carbohydrates such as cyclodextrins. Cyclodextrins are toroidal molecules consisting of 6, 7 or 8 glucose units (α-, β- and γ-cyclodextrin). The interior of the ring binds a hydrophobic tail of a molecule such as a surfactant. The resultant inclusion complex is generally formed with a 1:1 stoichiometry between surfactant and cyclodextrin. γ-Cyclodextrin and particularly α-cyclodextrin are preferred for use in this invention. Preferably enough sequestrant is used to be capable of sequestering or inactivating all the cell lysis reagent present. Preferably the amount of sequestrant is from 0.5 - 10%, particularly 1 - 5%, by weight on the weight of the reaction mixture.

It is an advantage of the invention that steps i), ii) and iii) can all preferably be performed in a single reaction vessel. The cells from which the analyte is extracted in step i) may be dead but are preferably living. It may be convenient to culture the cells in the reaction vessel in which the assay is to be performed. Preferably multiple assays are performed in parallel in wells of a multiwell plate such as a microtitre plate.

Preferably the cell lysis fluid that results from step i) is used, without any intermediate separation or purification, for performing steps ii) and iii). Preferably the sequestrant is included in one of the reagents that is mixed with the cell lysis fluid in step ii). Thus the components present in

an assay according to the invention may typically comprise:

a)   a source of cells possibly, or suspected of, containing the analyte;

b)   a cell lysis reagent;

c)   an unlabelled specific binding partner of the analyte which is, or is capable of being, immobilised on a solid support;

d)   a specific binding partner, or an analogue, of the analyte, which is either labelled or unlabelled and capable of being labelled.

One or both of components c) and d) includes a sequestrant, the order of addition of components c) and d) being immaterial.

In one format of the invention, the immunoassay is a scintillation proximity assay. In this format, components a), b), c) and d) are contained in the wells of a microtitre well plate, component d) being a radioactively labelled analogue of the compound being tested for. The scintillation proximity assay measurement is initiated by the addition to the wells of SPA fluomicrospheres coated with a binding reagent such as a secondary antibody or protein A.

Alternatively, cells may be cultured in the wells of a scintillant microtitre well plate suitable for the purpose, the base and/or walls of the wells being coated with a binding reagent such as a secondary antibody or protein A. After a suitable time, the remaining assay components, b), c) and d) are added to the wells.

In a second format of the invention, the immunoassay is an enzyme-immunoassay. In this format, components a), b), c) and d) are contained in the wells of a microtitre well plate, component d) being an enzyme-labelled specific binding partner of the compound being tested for. The assay measurement is initiated by the addition to the wells of detection reagents suitable for the detection of the enzyme label.

Suitable sequestration reagents are chosen from the group consisting of complex carbohydrates, including cyclodextrins. In a preferred

format, alpha-cyclodextrin is employed in the method of this invention.

The method comprises incubating cells with cell lysis buffer, adding to the mixture of lysed cells, the labelled specific binding partner for the substance, followed by addition of the unlabelled specific binding

5   partner, both reagents being dissolved in a buffer containing sequestration agent, and measuring the signal generated by the labelled specific binding partner as a measure of the amount of substance or component, with the entire quantities of reagents present in the reaction vessel at the same time. The signal obtained may be compared with the signals obtained

10 using a set of standard quantities of substance using a parallel procedure and generating a standard curve for the assay.

In one format, the immunoassay process is a radioimmunoassay, in which the labelled specific binding partner contains a radioisotope. Suitable radioisotopes for use in the assay method of the

15 present invention include β-emitting isotopes such as tritium, and iodine-125, which emits Auger electrons.

In an alternative format, the immunoassay process is an enzyme-immunoassay, in which the labelled specific binding partner is, or can be, bound to an enzyme label. Typical enzyme labels suitable for use

20 in the present invention are alkaline phosphatase, β-galactosidase, horseradish peroxidase, malate dehydrogenase and glucose-6-phosphate dehydrogenase. Horseradish peroxidase is a particularly preferred enzyme label for use in the enzyme immunoassay method according to the present invention.

25 In another format, the labelled specific binding partner can include a fluorescence label. Suitable fluorescent labels for use in the present invention may be selected from fluorescein, rhodamine and cyanine dyes.

The precise assay format, choice of specific binding partner,

30 the detection label, and the nature of the substance to be tested for are not

critical to the present invention. Rather, the invention relies on the
unexpected observation that a precise measurement of intracellular
components can be made without the separate extraction and/or
purification procedures being performed on the cell samples which are

5   inherent in, and characterise the prior art methods.

Illustrative of the immunoassay methods which can be
utilised in the present invention are the following assay formats.

I)   Radioactive Assays
10   ## Scintillation Proximity Assay using Scintillant Beads

The method provides a simple, single-step lysis and
measuring method for intracellular components. The immunoassay
reagents (antisera, tracer, SPA beads) are added to the same wells which
are used for growing cells. The process is carried out in single wells

15   without further technical intervention. In this aspect of the invention there is
a requirement for cultured cells grown in a suitable vessel. In a preferred
form of the invention there is a requirement for a tissue-culture treated
microtitre plate with opaque walls and a clear base, to allow microscopic
inspection of the cells. A lysis reagent is added to the cultured cells,

20   followed by labelled specific binding partner, unlabelled specific binding
partner and second antibody derivatised scintillant beads prepared in
buffer containing the sequestration agent. Standards are added to empty
microtitre wells on the same plate. The plate is incubated for a suitable
time period before counting on a β-scintillation counter. The concentration

25   of analyte in the samples is determined by interpolation from a standard
curve.

Alternatively, following the lysis event, a specific binding
partner coupled to scintillant beads is incubated with the antigen, together
with a second specific binding partner. The second binding partner is

30   unlabelled and detection is through a third binding reagent which is

labelled.

Alternatively, following the lysis event, the antigen/second specific binding partner complex is bound to the scintillant beads, the second specific binding partner being unlabelled and detection is through a third binding reagent which is labelled.

Scintillation Proximity Assays using Scintillating Microtitre Plates

In an alternative for of the assay system described above, a Cytostar-T plate (or equivalent) is used. In a preferred embodiment of the invention, there is a requirement for a sterile, tissue-culture-treated scintillant microtitre plate with opaque walls and a clear base to allow microscopic inspection of the cells. In this method, the plate is pre-coated with specific or secondary antibodies. As above, this method provides a simple, single-step lysis and measuring method for intracellular components of cultured cells grown in the Cytostar-T plate (or equivalent). The immunoassay reagents (tracer, +/- antisera) are added to the same wells which are used for growing cells. A lysis reagent is added to the cultured cells, followed by tracer (or antisera depending on whether primary or secondary antibodies have been used to coat the plate) dissolved in buffer containing the sequestration reagent. Standards are added to empty wells on the same plate. The plate is incubated for a suitable time period before counting on a β-scintillation counter. The concentration of analyte in the sample is determined by interpolation from a standard curve.

II Enzyme Immunoassays

'EMIT' type (Rubenstein KE. et al, 1972. Biochem. Biophys. Res. Comm. 47; 846)

The enzymes malate dehydrogenase and glucose-6-phosphate dehydrogenase have been used extensively in the

homogeneous immunoassay exemplified by the EMIT (Enzyme Multiplied Immunoassay Technique) system. Both enzymes are monitored by the conversion of the cofactor NAD to $NADH_2$ in a spectrophotometer at 340nm. In this assay system, the analyte competes with labelled antigen

5   for antibody binding sites. The activity of the enzyme is modified when the antibody binds to the labelled antigen.

Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous EMIT EIA are added dissolved in buffer containing the sequestration

10   (cyclodextrin) agent. Optical density is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

15   **'CEDIA' type** (Henderson DR *et al* 1986 Clin. Chem.32,1637-1641)

In the cloned enzyme donor immunoassay method, two inactive fragments of β-galactosidase have been synthesized by genetic engineering. The large fragment, the enzyme acceptor, contains 95% of the enzyme, and the small fragment, the enzyme donor, consists of the

20   remaining 5%. On mixing, the two fragments aggregate into tetramers which have β-galactosidase enzyme activity. In this assay, antigen is conjugated to the enzyme donor in such a way that aggregation with the enzyme acceptor is blocked if antibody binds to antigen. In the presence of analyte, less conjugate is bound by antibody, and enzyme activity is

25   stimulated. In the absence of analyte, antibody binding to the conjugate prevents formation of the active enzyme.

Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous CEDIA (antibody, enzyme-donor/ligand conjugate, enzyme acceptor

30   monomer) are added dissolved in buffer containing the sequestration

(cyclodextrin) agent. Optical density is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells

5

III Fluorescence Immunoassay Formats
Fluorescence polarization

Fluorescence polarization is a technique used to distinguish from free analyte without the need for separation. In competitive assays,

10 for small molecules, fluorescent-labelled antigens (e.g. using fluorescein, rhodamine or cyanine dye reagents) as tracer. At the signal generation and detection stage, a fluorimeter generates vertically polarized light at the excitation wavelength of the fluorophore.

The emitted light, at a lower wavelength because of Stokes'

15 shift, is detected through a vertical polarizing filter. Because free tracer rotates at a very high speed, the emitted light is always in a different plane from the incident light, so the amount of light detected through the polarizing filter is minimal. However the tracer bound the much larger antibody molecule is restrained from rotating at such a high speed and the

20 emitted light is in almost the same plane as the incident light. Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components of the homogeneous fluorescence assay (antibody, fluorescent-tagged antigen) are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Fluorescence is

25 measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

Fluorescence resonance energy transfer (FRET)

Different fluorophores often have different activation and emission spectra (see Table 2). However, the activation peak of one fluorophore may overlap with the emission peak of another fluorophore. if
5 the second fluorophore is placed in the immediate vicinity of the first, quenching of the fluorescent emission takes place through transfer of energy. This principle has been used in FRET by coupling one fluorophore to antibody and another to antigen. Binding of the antigen to antibody leads to close proximity and subsequent quenching. An alternative system
10 involves two populations of antibodies raised against the same antigen, labelled with two different fluorophores. Binding of the two antibodies gives rise to close proximity and then energy transfer between the two leading to quenching or reduction in fluorescence. Cells are cultured in a sterile, clear tissue culture treated microtitre plate, lysed and then the other components
15 of the homogeneous fluorescence are added dissolved in buffer containing the sequestration (cyclodextrin) agent. Fluorescence is measured and the concentration of analyte in the samples is determined by interpolation from a standard curve. Standards are added to empty wells of the same plate that are not used for growing cells.

20

Table 2. The spectral properties of Cyanine Fluorescent Dyes

Fluorophore	Activation (nm)	Emission (nm)
Cy2	489	506
Cy3	550	570
Cy3.5	581	596
Cy5	649	670
Cy5.5	675	694
Cy7	743	767
FluorX	494	520

Time-resolved fluorescence

Background fluorescence is one of the main problems with the use of fluorescence immunoassay, and its presence can severely limit the use of these methods. However, background fluorescence present in most biological material has the short lifetime of a few nanoseconds. For fluorophores with long fluorescence lifetimes, it is possible to measure fluorescence at a time when virtually all the background fluorescence has disappeared. This is essentially the principle of time resolved fluorescence and this method can be used in combination with fluorescence energy transfer and fluorescence polarization techniques.

IV)   Other methods

The method described in this patent can be applied to other diverse homogeneous (non-separation) immunoassay techniques such as luminescence, nephelometry, latex agglutination assays and their variants.

EXAMPLES

A. PRELIMINARY EXPERIMENTS

Scintillation proximity radioimmunoassay for adenosine 3'5' cyclic monophosphate

General Assay Conditions

Measurement of adenosine 3'5'cyclic monophosphate (cAMP) was selected as a model system for studying lysis and measurement of intracellular molecules. However, it is highly likely that the method described in this patent application is suitable for other intracellular analytes. A number of potential extractants were selected from among various surfactants known to lyse and liberate contents from eukaryotic

cells. These lysis reagents were used in a series of experiments in combination with sequestrating reagents in order to determine optimal reagents to be used. Standard curves for cAMP were prepared in lysis and sequestrating reagents. Parameters such as assay sensitivity,

5   standard curve working range and antigen: antibody binding were used to establish the most suitable reagents and optimal concentrations for both lysis and sequestrating agent. Standard curves were prepared as follows.

All assays were carried out in microtitre plates compatible with a microtitre plate beta scintillation counters. Standards (50μl; 0.2-

10   12.8pmol/well), antisera (50μl; at 1:11000 dilution), tracer (50ul; 10000-20000cpm) are added to anti-rabbit coated SPA beads (50μl; 20mg/ml). Non-specific binding was determined in the absence of specific rabbit antisera. SPA anti-rabbit reagent is placed onto a magnetic stirrer to ensure a homogeneous suspension before pipetting. All wells contained a

15   total volume of 200μl. The plates were sealed and incubated at room temperature (15-30°C) for 15-20 hours. The amount of [^{125}I]cAMP bound to the SPA fluomicrospheres was determined by counting in a microtitre plate beta scintillation counter for 2 minutes.

Experiments were set up with:-

20   a)   Working standards (4-256pmol/ml; 0.2-12.8pmol/well) were prepared in assay buffer (0.05M sodium acetate buffer containing 0.01% sodium azide) only (control) or assay buffer containing lysis reagent (e.g. DTAB, CPC or SDS; see experiment 1) at various concentrations.

b)   Rabbit anti-cAMP sera prepared in assay buffer only (control)

25   or assay buffer containing sequestrating agent at various concentrations depending on the experiment.

c)   Radioactive tracer: adenosine 3' 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester prepared in assay buffer only (control) or assay buffer containing sequestrating agent at various

30   concentrations depending on the experiment.

d)   Donkey anti-rabbit IgG coupled to scintillation proximity fluomicrospheres prepared in assay buffer only (control) or assay buffer containing sequestrating agent at various concentrations depending on the experiment.

5

Experiment 1: Inhibition of antigen and antibody binding by several lysis reagents

Method

10   In these experiments, standard curves were prepared for cAMP using the SPA immunoassay technique, described above, where working standards of cAMP were prepared in several lysis reagents at 1% (w/v) or 2% (w/v) final concentration. The detergents investigated included dodecyl trimethyl ammonium bromide (DTAB; Sigma Chemical Co. D8638), benzethonium chloride (BZC; Aldrich; B470-8), cetyl pyridinium chloride (CPC; Sigma Chemical Co; C9002), sodium dodecyl sulphate (SDS, Sigma chemical Co; L4509) and N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulphonate (DDAPS; Sigma Chemical Co.; D4516).

Results

Table 3a). The effect of several lysis reagents on antibody:antigen binding (zero concentration of sequestrant). These data shown are counts per minute (cpm) obtained on a TopCount™ microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standard (pmol cAMP/well)	Control (no lysis reagent)	1% DTAB	1% BZC	1% CPC	1% SDS	1% DDAPS
NSB	113	130	111	121	118	181
Zero	2674	1429	951	466	1149	1109
0.2	2068	1118	644	270	1052	969
0.4	1952	1098	501	239	973	849
0.8	1770	1088	473	232	913	725
1.6	1463	977	426	147	859	610
3.2	1193	860	261	125	837	425
6.4	862	649	16	103	640	311
12.8	458	440	6	80	394	204

Table 3b). The effect of increased concentrations of several lysis reagents on antibody : antigen binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	2% CPC	2% BZC	2% DTAB	2% DDAPS
NSB	117	133	117	145	129
Zero	2615	173	486	272	579
0.2	2100	133	308	231	416
0.4	1937	128	338	231	426
0.8	1786	108	222	201	429
1.6	1466	82	156	195	339
3.2	1202	77	143	179	303
6.4	867	73	96	170	215
12.8	459	50	70	93	109

Discussion

These data clearly demonstrates a significant reduction in antigen:antibody binding (cpm) where lysis reagents were included in the cAMP immunoassay.

Experiment 2: Restoration of antigen and antibody binding with the addition of sequestrant (2.5% alpha-cyclodextrin).

Method

In these experiments, standard curves for cAMP were prepared using the SPA immunoassay technique described above, where working standards of cAMP were prepared in 1% (w/v), 1.5% (w/v) or 2% (w/v) lysis reagents (DTAB, SDS or CPC). The control consisted of no lysis reagent or cyclodextrin added. The effect including 2.5% (w/v) alpha-cyclodextrin (alpha-CD) (Sigma Chemical Co.; C4642) on antigen:antibody binding was investigated. Here, tracer, antisera and SPA beads were prepared in assay buffer containing 2.5% (w/v) alpha-CD. A second control tested the effect of 2.5% (w/v) alpha-CD on the standard curve without addition of lysis reagent.

Results

Table 4a). The effect of lysis reagent (1% DTAB) on antigen
: antibody binding with or without the addition of sequestrant. These data
shown are counts per minute (cpm) obtained on a TopCount microplate
5   scintillation counter. Efficiencies of other multihead beta counters vary
from this and may give different results. Non-specific binding (NSB) data
was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No lysis reagent)	1% DTAB	2.5% CD only	1% DTAB 2.5% CD
NSB	186	182	155	140
Zero	3504	1586	3300	3361
0.2	3205	1575	2790	2931
0.4	2861	1573	2485	2638
0.8	2576	1305	1979	2228
1.6	1948	1097	1503	1724
3.2	1461	813	1058	1169
6.4	990	619	674	811
12.8	596	439	415	520

Table 4b). The effect of lysis reagent (1% SDS) on antigen : antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	1% SDS	2.5% cyclodextrin only	1% SDS plus 2.5% cyclodextrin
NSB	167	118	199	163
Zero	3147	1149	3145	3261
0.2	2335	1128	2358	2374
0.4	2179	1052	1979	2040
0.8	2080	973	1682	1851
1.6	1665	913	1499	1463
3.2	1269	859	1143	1100
6.4	857	640	754	813
12.8	411	394	383	416

Table 4c). The effect of lysis reagent (1 and 2% CPC) on antigen:antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control (No detergent)	1% CPC	1% CPC 2.5% CD	2% CPC	2% CPC 2.5% CD
NSB	118	121	115	134	121
Zero	2615	466	2122	173	779
0.2	2100	270	1650	133	455
0.4	1937	239	1534	128	431
0.8	1786	232	1541	108	360
1.6	1466	147	1269	82	329
3.2	1202	125	1017	77	206
6.4	867	103	735	73	139
12.8	459	80	393	50	79

Table 4d). The effect of several lysis reagents on antigen:antibody binding with or without the addition of sequestrant. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control	1% DDAPS	1% DDAPS 2.5% CD	2% DDAPS	2% DDAPS 2.5% CD	2% DTAB	2% DTAB 2.5% CD
NSB	121	137	142	130	137	138	132
Zero	2989	906	2179	510	738	217	741
0.2	2453	762	1792	423	579	198	578
0.4	2279	719	1724	370	613	216	566
0.8	2072	689	1612	350	543	201	579
1.6	1734	559	1342	290	413	179	471
3.2	1346	446	1153	252	351	176	403
6.4	916	326	823	194	262	144	313
12.8	600	173	453	115	140	78	190

Table 4e). The effect of varying concentrations of alpha cyclodextrin on antibody:antigen binding in the presence of 1.5% lysis reagent. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/well)	Control	1.5% DTAB only	1.5% DTAB 2.5% CD	1.5% DTAB 3% CD
NSB	239	237	177	165
Zero	4623	819	4430	4306
0.2	4165	799	4069	3855
0.4	3612	713	3559	3629
0.8	3257	688	3369	2966
1.6	2550	513	2510	2356
3.2	1951	426	1991	1662
6.4	1368	376	1341	1220
12.8	922	264	849	753

Discussion

These data clearly demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis reagents were added to the cAMP standards and where tracer, antisera and SPA beads were prepared in sequestration agent. Alpha-CD only had little impact on the standard curve.

Experiment 3: The optimal concentration of sequestrant

<u>Method</u>

In this experiment, standard curves for cAMP were prepared using the SPA radioimmunoassay where working standards of cAMP were prepared in 1% (w/v) DTAB. The effect of including 1% (w/v), 2% (w/v), 2.5% (w/v) and 5% (w/v) alpha-cyclodextrin on antigen:antibody binding was investigated. Here, tracer, antisera and SPA beads were prepared in assay buffer containing alpha-CD at the above concentrations. The control consisted of no lysis or cyclodextrin added to the assay.

<u>Results</u>

Table 5). The effect of lysis reagent and varying concentrations of sequestrant antigen : antibody binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/ well)	Control	1% DTAB only	1% DTAB 1% CD	1% DTAB 2% CD	1% DTAB 2.5% CD	1% DTAB 5% CD
NSB	172	130	121	179	165	140
Zero	3315	1429	1914	3250	3460	2980
0.2	3182	1118	1478	2849	3056	2490
0.4	2732	1098	1305	2525	2674	2145
0.8	2318	1088	1138	2143	2246	1800
1.6	1839	977	920	1639	1706	1372
3.2	1360	860	685	1245	1175	997
6.4	882	649	421	878	853	649
12.8	610	440	179	515	506	421

Discussion

These data clearly demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis reagents were added to the cAMP standards and where tracer, antisera and SPA beads were prepared in sequestration agent. 1% (w/v) and 2% (w/v) alpha-CD restored binding to a limited degree. 5% (w/v) alpha-CD was inhibitory. 2.5% (w/v) alpha-CD gave optimal results.

Experiment 4: The optimal sequestrant

Method

In this experiment, standard curves for cAMP were prepared using the SPA immunoassay technique where working standards were prepared 1% (w/v) lysis reagent (DTAB). The effect of including 2.5% (w/v) alpha-cyclodextrin (CD) 2.5% (w/v) beta-cyclodextrin (beta-CD) (Sigma Chemical Co.; C4767) and 2.5% (w/v) gamma-cyclodextrin (gamma-CD) (Sigma Chemical Co.; 4892) on antigen:antibody binding was investigated. Here, tracer, antisera, and SPA beads were prepared in assay buffer containing the appropriate sequestration reagent. The control consisted of no lysis agent or cyclodextrin added to the assay.

Results

Table 6). The effect of adding different sequestrants on antigen:antibody binding. These data shown are counts per minute (cpm) obtained on a TopCount microplate scintillation counter. Efficiencies of other multihead beta counters vary from this and may give different results. Non-specific binding (NSB) data was obtained in the absence of specific rabbit antisera.

Standards (pmol cAMP/well)	Control	1% DTAB	1% DTAB 2.5% alpha CD	1% DTAB 2.5% beta CD	1% DTAB 2.5% gamma CD
NSB	212	130	173	103	172
Zero	4276	1429	4135	3225	4295
0.2	3752	1118	3604	2664	3650
0.4	3411	1098	3229	2360	3322
0.8	2788	1088	2548	1755	2270
1.6	2100	977	2005	1304	2066
3.2	1588	860	1480	966	1413
6.4	1104	649	1043	658	966
12.8	692	440	498	322	532

Discussion

The data demonstrates a significant restoration in antigen:antibody binding (cpm) where lysis agent was added to the cAMP standards, and where tracer, antisera and SPA beads were prepared in each of the sequestration reagents. Alpha and gamma cyclodextrin restored binding to an optimal degree, whereas beta-cyclodextrin was less effective.

Overall Conclusions

These preliminary experiments established the utility of dodecyl trimethyl ammonium bromide (DTAB) as the preferred surfactant for cell lysis.

Similarly, in preliminary experiments, several sequestration reagents were evaluated including alpha-cyclodextrin, beta-cyclodextrin and gamma-cyclodextrin. Alpha-cyclodextrin was established as the preferred sequestration reagent.

B. THE OPTIMISED ASSAY SYSTEM

Experiment 5: Single-step extraction and measurement of adenosine 3', 5' cyclic monophosphate from forskolin-stimulated Chinese hamster ovary cells

Reagents, buffers and equipment

The following solutions were prepared:

1)   Assay buffer; 0.05M sodium acetate buffer containing 0.01% (w/v) sodium azide.

2)   Lysis reagent; DTAB (10% w/v) dissolved in assay buffer (useful for non-adherent cell lines).

3)   Lysis reagent; DTAB (1% w/v) dissolved in assay buffer (useful for adherent cell lines such as Chinese Hamster Ovary Cells, this example) .

4)   Sequestration reagent; alpha-cyclodextrin (2.5%w/v) dissolved in assay buffer.

5)   Adenosine 3', 5' cyclic monophosphate (cAMP) standard; 512pmol, for the assay, used in the range 0.2-12.8pmol/well, prepared in 2ml of assay buffer containing 1%(w/v) DTAB to give 256pmol/ml (see below).

6)   Radioactive tracer: adenosine 3', 5'-cyclic phosphoric acid 2'-O-succinyl-3-[^{125}I] iodotyrosine methyl ester prepared in assay buffer containing 2.5% alpha-cyclodextrin.

7)   Rabbit anti-cAMP sera prepared in assay buffer containing 2.5% alpha-cyclodextrin.

8)   Donkey anti-rabbit IgG coupled to scintillation proximity fluomicrospheres prepared in assay buffer containing 2.5% alpha-cyclodextrin.

Additional materials and equipment required are as follows.

i)   Clear-bottomed microtitre plates with opaque walls, tissue culture treated plates (e.g.. Viewplates™, Packard or Cytostar-T plates, Amersham).

ii)   Microplate scintillation counter.

iii)   Plate sealers.

iv)   Disposable polypropylene or polystyrene test tubes for preparing working standards.

v)   Pipettes and pipetting equipment.

vi)   Laboratory glassware.

vii)   Distilled water.

viii)   Vortex mixer.

ix)   Magnetic stirrer.

x)   1% (w/v) trypan blue solution prepared in water.

xi)   Haemocytometer

xii)   HAM's culture media

xiii)   Dimethylsulphoxide

xiv)   Forskolin

xv)   Cultured Chinese hamster ovary (CHO) cells at approximately 10^6 cells/ml.

Method

Chinese hamster ovary cells were grown in HAM's media containing 10% (v/v) foetal calf serum (FCS). For cAMP assays, cells were seeded into 96-well tissue culture plates (see materials) in HAM's media,

5   as above, at 100µl/well (10^5 cells/well). The cells were cultured overnight at 37°C in a 95%air/5%CO_2 atmosphere. The next day, sequestration and lysis buffers (2.5% alpha-cyclodextrin and 1% DTAB in assay buffer) were prepared. Antisera (at 1/11000 dilution), tracer (10000-20000 cpm) and SPA beads (20mg/ml) were prepared in assay buffer containing 2.5%

10   alpha-cyclodextrin. Working standards (4-256pmol/ml; 0.2-12.8pmol/microtitre well) were prepared in polypropylene tubes using assay buffer containing 1% DTAB. Fifty microlitres aliquots of each working standard were added to empty of wells of the same microtitre plate used for culturing cells. Forskolin (1mg) (to stimulate cAMP generation)

15   was dissolved in DMSO (1ml), and diluted with HAM's media containing 10% FCS, to give various concentrations of forskolin from 1µM to 10µM. Two assay controls (blanks) were prepared in a similar manner. These consisted of a DMSO (assay) blank and a culture media (sample) only blank. The forskolin and blank solutions (100µl) were added to the

20   cultured cells, and incubated at 37°C, as above, for 20 minutes. At this stage, a check was made that the cells remained viable using a trypan blue exclusion test. The culture media was aspirated from the stimulated walls (the cells remain adhered to the surface of the culture vessel), and the incubation terminated by the addition of lysis reagent (1% DTAB dissolved

25   in assay buffer). A check for cell lysis was made with a second trypan blue exclusion test.

Fifty microlitres of primary antibody, tracer, and SPA beads (prepared in buffer containing 2.5% (w/v) alpha CD) , were added to standards and the samples in the 96-well culture plate. The plates were

30   sealed and incubated overnight at room temperature. Following

incubation, the plates were transferred directly to a TopCount™ scintillation counter and radioactivity detected. Non-specific binding was determined in the absence of specific rabbit antisera. Cyclic AMP levels were determined using log/linear analysis with reference to the standard curve. Levels were
5   estimated by interpolation.

This method is readily modified for extraction and measurement of cAMP in non-adherent cells (e.g.. HL60 cells). For this modified procedure, 10% (w/v) lysis reagent (10% DTAB dissolved in assay buffer) is added to the cultured cells to give a final concentration of
10   1% (w/v). The remainder of the method is as described above.

Results

Dose-response curves were prepared for the one-step *in situ* measurement of cAMP from forskolin stimulated CHO cells.
15   Representative standard curves prepared on Viewplates and Cytostar-T plates are shown figures 1 and 2 respectively. In figure 3 , the effect of 1, 5 or 10μM forskolin on intracellular cAMP levels as measured by the one-step *in situ* measurement method on cells grown in 96-well plates is presented. CHO cells were seeded at a density of 100,000 cells/well and
20   grown overnight in 96-well plates as described in the methods section. Cells were exposed to various concentrations of forskolin for 20 minutes and cAMP levels measured as described above. Basal levels of cAMP were approximately 10pmol/10^6 cells in the absence of added forskolin and were augmented by the increasing concentrations of forskolin.

25

Discussion

These data illustrate the utility of the invention described in this patent application, whereby intracellular molecules are measured in a concerted one-pot lysis and estimating immunoassay system. Indeed, the

5   data presented in Figure 3 demonstrates lysis and measurement of cAMP in forskolin-stimulated Chinese Hamster Ovary cells. The estimated levels of this cyclic nucleotide is in accordance with other published data whereby cAMP was determined by a more complex procedure (see Hancock *et al*, 1995). Furthermore, we have accumulated results in separate experiments

10   whereby cAMP levels were measured with the method described in this patent application and compared with levels extracted and subsequently estimated with a traditional procedure (ethanol extraction procedure, Horton & Baxendale, 1995) (data not shown). The results, obtained with these two different methods, gave highly similar values in levels of

15   intracellular cAMP.

LEGEND TO FIGURES

Figure 1. **Representative standard curve for the concerted one-pot extraction and immunoassay method for the**

20   **estimation of adenosine 3'5' cyclic monophosphate from cultured Chinese Hamster Ovary cells.**

Assay curves were prepared on Viewplates (Packard) as described in the methods section.

The percent B/B_0 function was calculated from the following

25   formula:-

[(standard counts per minute)-(non-specific binding counts per minute)] divided by [(zero standard counts per minute)-(non-specific binding counts per minute)] multiplied by 100%.

Typically, counts per minute values for the zero dose were

30   2200. The counts per minute values obtained in the absence of specific

antibody (non-specific binding wells) were approximately 200. The values in the absence of tracer were usually less than 25cpm.

Figure 2. Representative standard curve for the concerted one-pot extraction and immunoassay method for the
5 **estimation of adenosine 3'5' cyclic monophosphate from cultured Chinese Hamster Ovary cells.**

Assay curves were prepared on Cytostar-T as described in the methods section.

The percent B/B_0 function was calculated from the following
10 formula:-

[(standard counts per minute)-(non-specific binding counts per minute)] divided by [(zero standard counts per minute)-(non-specific binding counts per minute)] multiplied by 100%.

Typically, counts per minute values for the zero dose were
15 2200. The counts per minute values obtained in the absence of specific antibody (non-specific binding wells) were approximately 200. The values in the absence of tracer were usually less than 25cpm.

Figure 3. Forskolin-stimulated cAMP generation from cultured Chinese Hamster Ovary Cells as determined by the one-pot
20 **extraction SPA radioimmunoassay method.**

CHO cells were seeded at a density of 100,000 cells per well and grown overnight to confluence in clear-bottomed Cytostar-T or Viewplates as described under the methods section. Cells were exposed to various concentrations of forskolin and cAMP extracted and levels
25 determined with the SPA radioimmuunoassay procedure. Basal levels of cAMP were less than 10pmoles/10^6 cells which significantly increased on stimulation by forskolin.

CLAIMS

1.   A method of assaying for an analyte which method comprises the steps of:

i)   mixing a sample of cells possibly containing the analyte with a cell lysis reagent to provide a cell lysis fluid,

ii)   mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

iii)   and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

2.   A method as claimed in claim 1, wherein the cell lysis reagent is a detergent.

3.   A method as claimed in claim 1 or claim 2, wherein the sequestrant is a cyclodextrin.

4.   A method as claimed in claim 3, wherein the amount of sequestrant is in the range of 1 - 5% of the binding reaction mixture.

5.   A method as claimed in any one of claims 1 to 4, wherein steps i), ii) and iii) are all performed in a single reaction vessel.

6.   A method as claimed in any one of claims 1 to 5, wherein multiple assays are performed in parallel in wells of a multiwell plate.

7.   A method as claimed in any one of claims 1 to 6, wherein the assay of step ii) is a homogenous assay.

8.   A method as claimed in claim 7, wherein the assay of step ii) is a scintillation proximity assay.

9.   A method as claimed in any one of claims 1 to 8, wherein the specific binding assay of step ii) is an immunoassay.

10.   A method as claimed in any one of claims 1 to 9, wherein the analyte is adenosine-3',5'-cyclic monophosphate, the cell lysis reagent is dodecyl trimethyl ammonium bromide and the sequestrant is α-cyclodextrin.

ABSTRACT

IN-SITU CELL EXTRACTION AND ASSAY METHOD

A method of assaying for an analyte comprises the steps of:

i)   mixing a sample of cells possibly containing the analyte with a cell lysis reagent to provide a cell lysis fluid,

ii)   mixing the cell lysis fluid with reagents, including a specific binding partner of the analyte for binding to the analyte, for performing a specific binding assay for the analyte,

iii)   and mixing the cell lysis fluid with a sequestrant for the cell lysis reagent, whereby the binding of step ii) is performed in the presence of the sequestrant.

Figure 1. Typical standard curve for the one-step extraction and immunoassay method for the estimation of cAMP from cultured CHO cells, (Viewplate data).
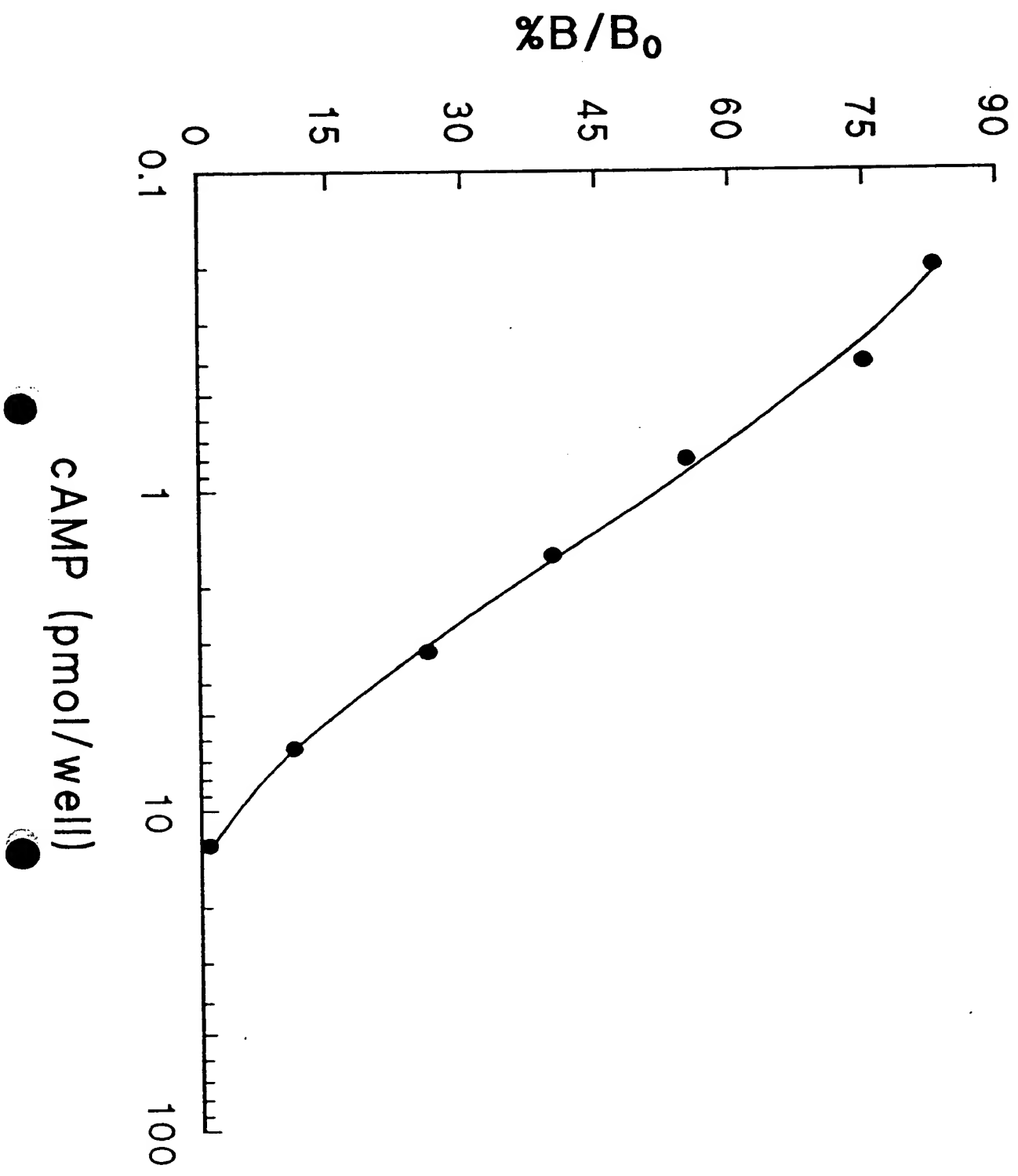
Figure 2. Typical standard curve for the one-step extraction and immunoassay method for the estimation of cAMP from cultured CHO cells, (Cytostar-T data).
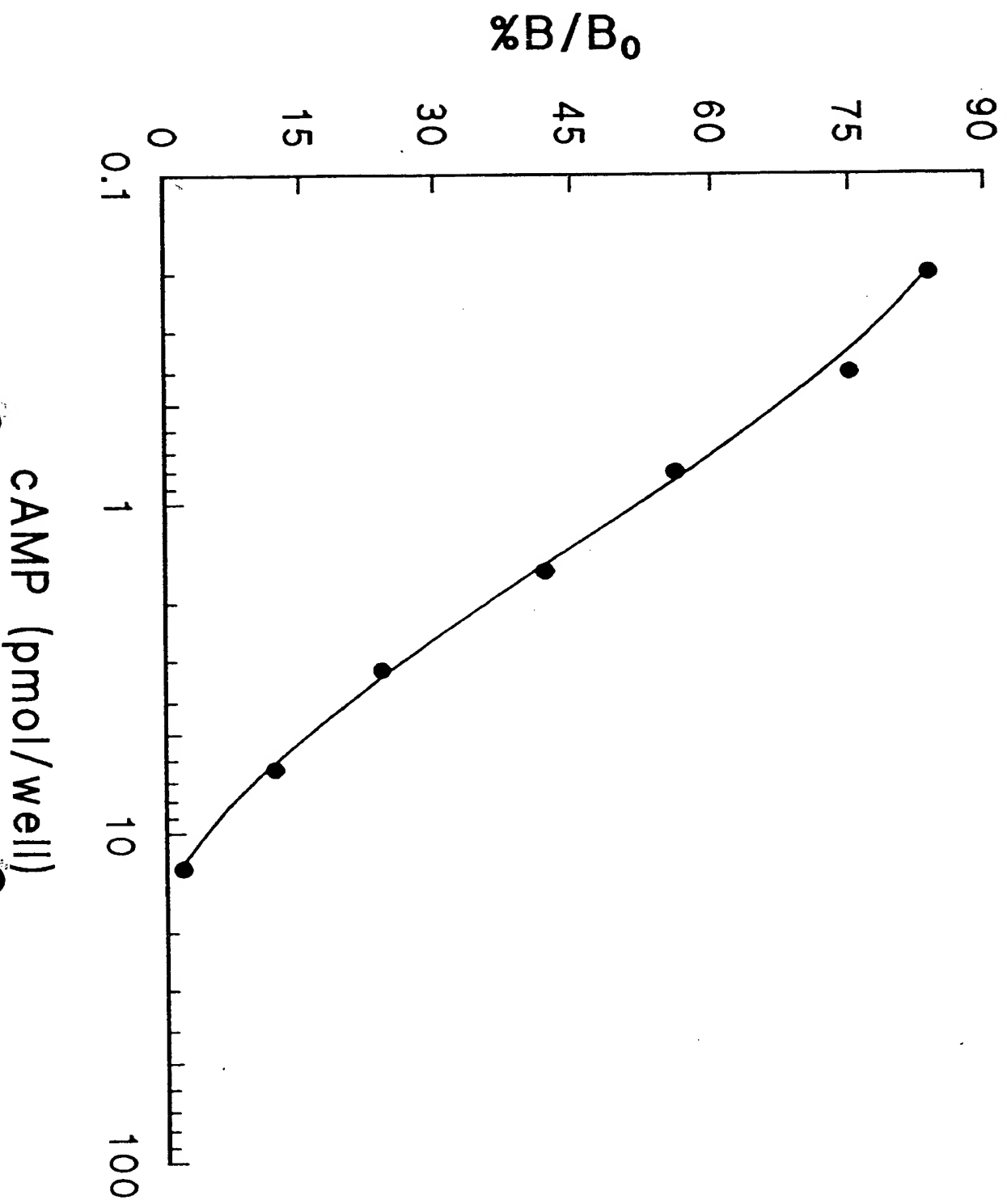
Figure 3. Forskolin-stimulated cAMP generation from CHO cells.